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# Activation of Electroneutral K Flux in *Amphiuma* Red Blood Cells by *N*-ethylmaleimide

## *Distinction between K/H Exchange and KCl Cotransport*

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**ABSTRACT** Exposure of *Amphiuma* red blood cells to millimolar concentrations of *N*-ethylmaleimide (NEM) resulted in net K loss. In order to determine whether net K loss was conductive or was by electroneutral K/H exchange or KCl cotransport, studies were performed evaluating K flux in terms of the thermodynamic forces to which K flux by the above pathways should couple. The direction and magnitude of the NEM-induced net K flux did not correspond with the direction and magnitude of the forces relevant to K conductance or electroneutral KCl cotransport. Both the magnitude and direction of the NEM-activated K flux responded to the driving force for K/H exchange. We therefore conclude that NEM-induced K loss, like that by osmotically swollen *Amphiuma* red blood cells, is by an electroneutral K/H exchanger. In addition to the above studies, we evaluated the kinetic behavior of the volume- and NEM-induced K/H exchange flux pathways in media where Cl was replaced by SCN, NO<sub>3</sub>, para-aminohippurate (PAH), or gluconate. The anion replacement studies did not permit a distinction between K/H exchange and KCl cotransport, since, depending upon the anion used as a Cl replacement, partial inhibition or stimulation of volume-activated K/H exchange fluxes was observed. In contrast, all anions used were stimulatory to the NEM-induced K loss. Since, on the basis of force-flow analysis, both volume- and NEM-induced K loss are K/H exchange, it was necessary to reevaluate assumptions (i.e., anions serve as substrates and therefore probe the translocation step) associated with the use of anion replacement as a means of flux route identification. When viewed together with the force-flow studies, the Cl replacement studies suggest that anion effects upon K/H exchange are indirect. The different anions appear to alter mechanisms that couple NEM exposure and cell swelling to the activation of K/H exchange, as opposed to exerting direct effects upon K and H translocation.

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## INTRODUCTION

Osmotic swelling or shrinkage stimulates volume-sensitive alkali metal and Cl fluxes in many cell types, which include the red blood cells of *Amphiuma* (Cala, 1980), duck (Kregenow, 1977), dog (Parker, 1983*a, b*), and sheep (Ellory and Dunham, 1980), Ehrlich ascites tumor cells (Hendil and Hoffman, 1974), and human lymphocytes (Grinstein et al., 1982, 1984). The volume-sensitive net ion fluxes of the above cells facilitate at least partial restoration of cell volume. Consequently, volume-sensitive flux pathways have been viewed as serving a volume-regulatory function.

Depending on the cell type, volume-sensitive alkali metal flux can occur by conductive or electroneutral pathways. On the basis of electrical measurements and later on thermodynamic criteria (Cala, 1980, 1983, 1985*a*), the volume-sensitive alkali metal flux pathways in *Amphiuma* red blood cells were shown to be electroneutral (see also Parker and Castranova, 1984). Osmotic shrinkage of the *Amphiuma* red blood cell activates Na/H exchange, while osmotic swelling activates K/H exchange (Cala, 1980). After cell shrinkage in hyperosmotic media or exposure to norepinephrine in isosmotic media, duck red blood cells gain Na, K, and Cl (Schmidt and McManus, 1977*a, b*). Subsequently, Haas et al. (1982) were able to demonstrate that after cell shrinkage or norepinephrine exposure, Na, K, and Cl were obligatorily coupled and were insensitive to changes in the membrane voltage. On the basis of the above observations, the authors concluded that the pathway activated by cell shrinkage or norepinephrine was electroneutral Na + K + 2Cl cotransport. In contrast to the electroneutral flux pathways of *Amphiuma* and duck red blood cells, net K and Cl fluxes after swelling of human lymphocytes or Ehrlich ascites tumor cells occur by activation of separate K- and Cl-conductive pathways (Grinstein et al., 1982; Hoffman et al., 1984).

A common property of all of the volume-sensitive alkali metal flux pathways described above is their anion sensitivity. For example, Cl or Br are obligatory substrates for volume-sensitive alkali metal ion flux in duck red blood cells. Consequently, replacing Cl or Br with other anions inhibits volume-sensitive ion flux (Haas et al., 1982). In contrast, Cl is not an obligatory, but rather is a functional, substrate for conductive K fluxes like those observed in studies of Ehrlich tumor cells or lymphocytes. When the Cl in Ehrlich ascites tumor cells or lymphocytes is replaced with an anion whose conductive permeability is lower than that of Cl (i.e., PO<sub>4</sub>, SO<sub>4</sub>), the rate of net K flux is retarded. However, when Cl is replaced by an anion with a higher conductive permeability than Cl (i.e., SCN, NO<sub>3</sub>), the rate of conductive K loss is increased owing to electrical effects (see Hunter, 1977; Grinstein et al., 1982; Parker, 1983*a*). Both the volume- and *N*-ethylmaleimide (NEM)-activated K fluxes of low-K sheep red cells are inhibited when Cl is replaced with other anions (Lauf, 1983, 1984, 1985; Louge et al., 1983). In this respect, the K flux from swollen and NEM-treated low-K sheep red blood cells is similar to the volume-sensitive electroneutral fluxes of the duck red blood cell. On the basis of the above, it was concluded that the volume- and NEM-activated K loss by low-K sheep red cells is electroneutral KCl cotransport (Lauf and Theg, 1980; Lauf, 1985).

The studies presented here demonstrate that NEM is able to activate net K

loss by *Amphiuma* red blood cells. In contrast to conclusions based upon studies of low-K sheep red blood cells, the NEM-induced K loss by *Amphiuma* red blood cells is shown to be by K/H exchange, as determined by force-flow analysis. Finally, data are presented evaluating the effects of anion replacement upon both NEM- and volume-activated K loss by *Amphiuma* red blood cells. The results of these studies suggest that the assumptions that anions are serving as electrical counterions for conductive transport or obligatory substrates for electroneutral flux pathways are not necessarily valid when studying inducible transport pathways. That is, rather than interacting with the ion flux pathways, anions may exert their primary effects upon the reaction(s) responsible for the activation of transport (see also Parker, 1984).

#### MATERIALS AND METHODS

The methods used for determination of Na, K, Cl, and water have been described previously (Cala, 1977). Blood was collected from healthy adult *Amphiuma* by cardiac puncture with a disposable syringe. Cells were washed three times in 30 vol of isotonic (control) Ringer solution containing (millimolar): 105 NaCl, 3 KCl, 1 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 6 HEPES, 12 Na-HEPES, 5 glucose, 1 ouabain, at pH 7.65 and 23°C. The red cells were then suspended at a hematocrit of ~10%, and incubated in control Ringer for 1.5 h at 23°C to ensure a stable ion and H<sub>2</sub>O content before experimental treatment. To begin an experiment, the cells were centrifuged from the incubation medium and resuspended at 10% hematocrit in the appropriate experimental medium. All experimental media contained [<sup>14</sup>C]inulin for the measurement of extracellular trapped medium. At appropriate time intervals, 375-μl samples were removed, placed in 400-μl polyethylene tubes, and centrifuged (model 3200, Brinkmann Instruments, Inc., Westbury, NY) for 2 or 4 min at 12,000 g. After centrifugation, the supernatant was removed from the cell pellet, and both were saved for analysis. The cell pellet was weighed, after which 230 μl of double-distilled H<sub>2</sub>O containing 4 mM MgSO<sub>4</sub> was added. This was followed by mechanical agitation, disrupting the cells and causing hemolysis. 20 μl of 0.5 M ZnSO<sub>4</sub> was added to the lysate in order to precipitate proteins. The lysate was subjected to centrifugation for 2 min at 12,000 g, which resulted in separation of a solid, insoluble pellet and a clear supernatant. The supernatant was then analyzed for Na, K, Cl, and [<sup>14</sup>C]inulin. The water-insoluble fraction of the cell lysate was dried to a constant weight in an oven for 24 h at 80°C, and the dry weight was determined.

#### *Cellular Ion and H<sub>2</sub>O Content*

Aliquots of the supernatant were taken for analysis as follows: 100 μl for Cl determination (Buchler Chloridometer, Instrument Division, Searle Diagnostics, Inc., Fort Lee, NJ); 50 μl for Na and K flame photometry (model 343, Instrumentation Laboratory, Inc., Lexington, MA); and 40 μl for determination of [<sup>14</sup>C]inulin by liquid scintillation counting (Beckman liquid scintillation system, Searle Diagnostics, Inc.). The values obtained from ion determinations and liquid scintillation counting, along with the wet and dry cell weights, were then used to calculate the cell ion and water content (and ion concentrations) after correction for extracellular ion and H<sub>2</sub>O contamination (<5%).

#### *Experimental Media*

Alterations in medium osmolarity were accomplished by changing [NaCl] unless otherwise specified. All media, both incubation and experimental, contained 1 mM ouabain. When the anion exchange inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) (Calbi-

ochem-Behring Corp., San Diego, CA) was used, it was prepared and added to the experimental media immediately before initiating the experiment. Similarly, NEM (500 mM in dimethylsulfoxide) (Sigma Chemical Co., St. Louis, MO) was made fresh and added to the experimental Ringer immediately before the start of an experiment.

#### *Electrical Measurements*

*Amphiuma* red cell membrane potential measurements were made by impaling cells with glass microelectrodes as previously described (Cala, 1980, 1983b).

#### *Measurement of Net K Fluxes*

Net K flux, expressed as millimoles K per kilogram dry cell solids (dcs) per hour, was determined from differences in K content at  $t = 0$  and at  $t = 60$  min. Measurement of net K flux as a function of time showed that K fluxes under isosmotic conditions in the presence of NEM, valinomycin, or NEM plus valinomycin were linear during the course of the experiments.

#### *Determination of Intracellular pH*

Cellular pH was determined from the Cl distribution ratios and the known external pH (i.e.,  $H_i/H_o = Cl_o/Cl_i$ ; see Cala, 1983b). In experiments upon cells in media with low external Cl or (see Table I) in the presence of DIDS, cellular pH was determined directly by measuring the pH of a cell lysate obtained by freezing and then thawing the cell pellet.

#### *Anion Replacements*

Anion replacements in isosmotic (240 mosmol) or hyposmotic (135 mosmol) media were performed by substituting NaCl with the Na salts of  $NO_3$ , SCN, para-aminohippurate (PAH), or gluconate. All other components of the media were identical to controls. The cells were preincubated in the above isosmotic or isotonic Ringer for 1.5–2 before the initiation of flux studies. This was done to ensure that the cells were all at control volume at the start of the experiment, and that, in all cases, cell [Cl] was zero. In the case of SCN and  $NO_3$  replacements, the cells contain these ions. Since PAH is poorly transported on the anion exchanger, cells placed in PAH media first lose Cl for  $HCO_3$ ; this is followed by a slower exchange ( $t_{1/2} = \sim 2$  h) of cell  $HCO_3$  for extracellular PAH. Consequently, at the time of the experiment, the PAH-treated cells have an elevated  $[HCO_3]$  and  $pH_i$  ( $pH_i$  increased from 7.0 to a final value of 7.1–7.2). Gluconate is not transported on the anion exchanger. As a result, cells suspended in media with Cl replaced by gluconate shrink and alkalinize as they lose all Cl and gain  $HCO_3$ . In order to compensate for gluconate-induced cell shrinkage, the cells were allowed to regulate volume by Na/H exchange (Cala, 1980), which alkalinized  $pH_i$  to  $\sim 7.6$ , or, alternatively, cells were transferred to isotonic gluconate media (empirically determined).

### RESULTS

#### *Activation of Net K Flux by NEM; K and Cl Loss as a Function of [NEM]*

*Amphiuma* red cells suspended in ouabain-containing isosmotic Ringer were exposed to NEM concentrations in the range of 0.5–8 mM for 1 h and were subsequently analyzed for ion and water content. Fig. 1 shows that while 0.5 mM NEM depresses K and Cl loss, increasing [NEM] from 1 to 8 mM progressively stimulates net K and Cl loss, with no change in Na content (not shown). The net K loss at zero NEM is consistent with expectations for loss through the

"leak" pathway by cells exposed to ouabain (note that all media contain ouabain). As has been described for volume- and Ca-activated K loss by *Amphiuma* red cells (Cala, 1980, 1983b), the NEM-induced net K loss exceeds that of Cl and results in cell shrinkage owing to movement of osmotically obliged water. During the 60-min experimental period, cell water was reduced by 15–20% at 8 mM NEM and  $\leq 10\%$  at 4 mM NEM. The latter concentration was used in all subsequent experiments.

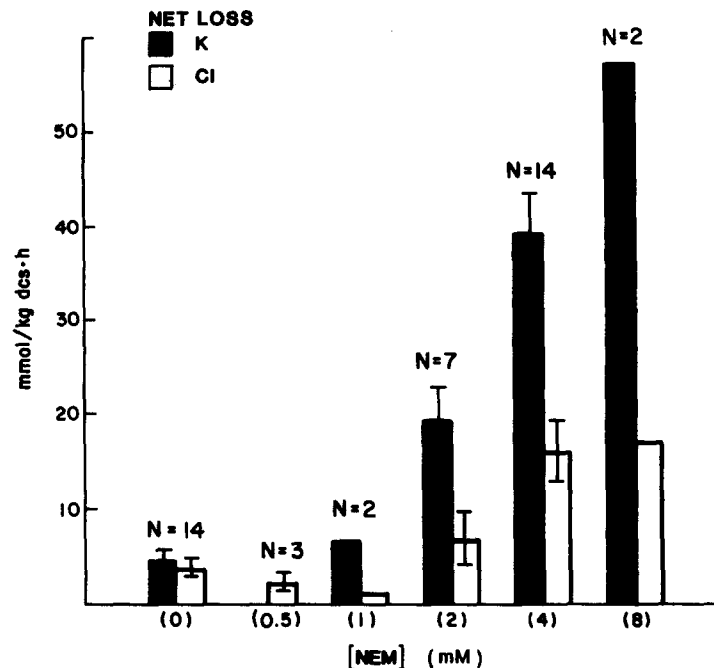


FIGURE 1. The change in cell K and Cl content (millimoles per kilogram dry cell solids [dcs]) during 1 h of exposure to 0, 0.5, 1, 2, 4, and 8 mM NEM. After preincubation in isosmotic Ringer (240 mosmol) for 1.5 h (as described in Materials and Methods), the experiment was initiated by resuspending the cells in fresh isosmotic media containing NEM at the concentration indicated. As stated in Materials and Methods, cell incubation and experimental media contain 1 mM ouabain. Data are expressed as means  $\pm$  SEM. The number of experiments (*N*) is indicated in the figure above the corresponding dose of NEM.

**Anion dependence of the NEM-induced K flux.** Studies to determine the nature of NEM-induced K loss, based upon the anion dependence of net K flux, were performed. In order to evaluate the anion sensitivity of volume- and NEM-induced K loss by *Amphiuma* red blood cells, medium Cl was replaced by NO<sub>3</sub>, SCN, PAH, or gluconate (>95% replacement). Fig. 2 shows the effects of Cl, NO<sub>3</sub>, SCN, PAH, and gluconate on NEM-induced (A) or volume-induced (B) net K flux. These data show that the volume- and NEM-induced net K fluxes have different anion sensitivities. Relative to Cl, the swelling-induced K loss is

inhibited by  $\text{NO}_3$  and  $\text{SCN}$ , stimulated by gluconate, and unaffected by PAH. In contrast, the NEM-induced K loss in isosmotic media was stimulated (relative to Cl) by all the anions tested. On the basis of anion dependence and the implicit assumption that anions exert their effects directly upon K translocation, we

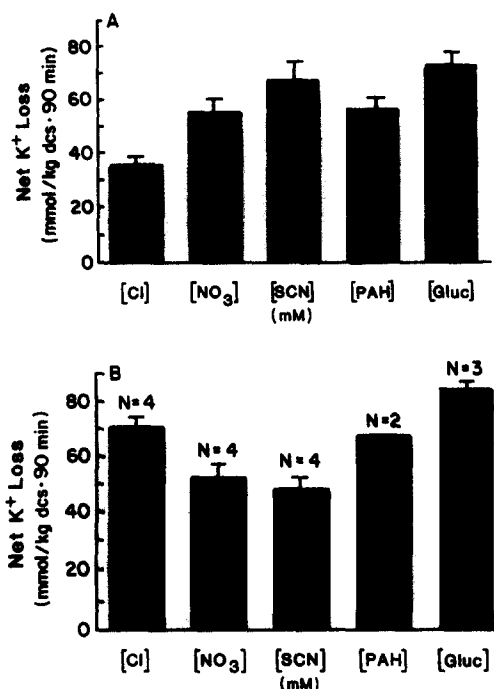


FIGURE 2. NEM- (A) or volume- (B) induced K loss by cells in media having the anion composition indicated. The cell Cl is zero in all media except Cl. Since  $\text{SCN}$  and  $\text{NO}_3$  move rapidly on the anion exchange, the cells suspended in  $\text{SCN}$  or  $\text{NO}_3$  media contain these anions. PAH transport by the anion exchange is slow ( $t_{1/2} > 120$  min), so cells in PAH medium lose Cl in exchange for  $\text{HCO}_3^-$  and become alkaline ( $\text{pH}_i \approx 7.2$ ). Because gluconate is not transported on the anion exchanger, the cells lose Cl for  $\text{HCO}_3^-$  in gluconate media. Consequently, the gluconate cells lose  $\text{H}_2\text{O}$  and have a  $\text{pH}_i$  of  $\sim 7.3$  (fixed  $P_{\text{CO}_2}$  with a well-buffered cell interior). This gluconate-induced shrinkage results in activation of the previously described volume-sensitive Na/H exchange, which causes the cells to gain Na and  $\text{H}_2\text{O}$ , returning to normal volume in  $\sim 1.5$  h (data not shown). This Na/H-dependent increase causes further alkalization to  $\text{pH}_i \approx 7.6$  at the time of the experiment. All cells are exposed to the test anion (in isotonic NEM-free medium) for 1.5–2 h before experimental treatment (swelling or NEM) to ensure that cell volume is the same regardless of media anion composition. Data are expressed as means  $\pm$  SEM.  $N = 5$  in A;  $N = <5$  in B.

would conclude that the NEM- and volume-activated net K fluxes are by different pathways. Using the same rationale as others, we would also conclude that the fraction of K loss from swollen cells that is inhibited by  $\text{NO}_3$  or  $\text{SCN}$  is Cl dependent and is therefore K plus Cl cotransport. This interpretation is at odds

with previous findings (Cala, 1980, 1985a), which showed that swelling-induced K loss by *Amphiuma* red cells is due solely to K/H exchange. Interpretation of the anion replacement studies is further confounded by the observation that  $\text{NO}_3$  and  $\text{SCN}$  (anions to which the membrane has a high conductance) stimulate NEM-induced K flux in a manner consistent with K flux by conductive routes. Contradicting this interpretation is the observation that the NEM-induced K loss is also stimulated by PAH and gluconate, anions to which the membrane has a low conductance relative to Cl.

Given the inconsistent effects of the anions on the NEM-induced K flux and the fact that Cl replacement with  $\text{SCN}$  and  $\text{NO}_3$  inhibited the volume-induced K/H exchange, it was clear that the anion substitution experiments per se would not suffice to define the mechanism of the NEM-induced K flux.

#### *The Nature of the NEM-induced K Flux*

**Possible modes of transport.** In light of the above difficulties, we investigated the mode of K loss based upon measurement of the membrane voltage. In an attempt to evaluate the relative contributions of conductive and electroneutral K flux pathways to the NEM-induced K flux, we measured the membrane potential ( $E_m$ ) of NEM-treated cells. As was the case for the volume-stimulated K loss (Cala, 1980), NEM exposure was without effect upon  $E_m$ . In addition, exposure of cells to the K ionophore valinomycin ( $2\ \mu\text{M}$ ) produced a net K loss of  $\sim 9\ \text{mM/kg dcs} \cdot 90\ \text{min}$  and an associated membrane hyperpolarization of 25 to approximately  $-50\ \text{mV}$  (the K equilibrium potential was approximately  $-90\ \text{mV}$ ), regardless of the presence or absence of NEM. The fact that the NEM did not alter the  $E_m$ , either in the presence or absence of valinomycin, illustrates that the compound did not change the K conductance relative to that of other charge carriers. The failure of NEM to alter the valinomycin-induced membrane hyperpolarization illustrates that the total membrane conductance is unchanged by the presence of NEM. Taken together, the  $E_m$  measurements argue that the NEM-induced increase in K loss is by electroneutral, as opposed to conductive, routes (see Cala, 1980, 1985a, b).

#### *Force-Flow Analysis*

**Distinction between conductive and electroneutral K flux.** The inability of NEM to change the membrane potential suggested that it may stimulate K flux via an electroneutral pathway. In order to test this hypothesis more rigorously, force-flow analysis was employed. Briefly, the net flux of any ion must couple to its appropriate driving force. If the NEM-induced net K flux is conductive, the magnitude and direction of the flux must correspond to the magnitude and direction (sign) of the electrochemical potential difference for K ( $\Delta\tilde{\mu}_K = \Delta\mu_K + zFE_m$ ). In contrast, if the K flux induced by NEM is K/H exchange, as is the case for osmotically swollen *Amphiuma* red blood cells, then the appropriate driving force is the difference between the K and H chemical potential differences ( $\Delta\mu_K - \Delta\mu_H$ ) (Cala, 1985a). Finally, if K flux is electroneutral by obligatorily coupled K plus Cl cotransport, the net K flux, magnitude, and direction will depend upon the magnitude and sign of the sum of the chemical potential difference for K and Cl ( $\Delta\mu_K + \Delta\mu_{\text{Cl}}$ ). Since, under normal conditions, H and Cl are at electro-



chemical equilibrium,  $-\Delta\mu_H = \Delta\mu_{Cl} = zFE_m$ ; therefore,  $(\Delta\mu_K - \Delta\mu_H) = (\Delta\mu_K + \Delta\mu_{Cl}) = (\Delta\mu_K + zFE_m)$ . If, however,  $E_m$  is altered by increasing the conductive permeability of some nonequilibrium-distributed ion,  $-\Delta\mu_H$  and  $\Delta\mu_{Cl}$ , while still equal, are no longer equal to  $zFE_m$  (Cala, 1983a, b, 1985a, b). Thus, by changing  $E_m$  with the K ionophore valinomycin, we can distinguish between conductive and electroneutral K flux but not between K/H exchange and K plus Cl cotransport.

Fig. 3 shows the results of the force-flow analysis for conductive and electroneutral net K flux. The data in Fig. 3 depict the NEM-induced net K flux as a

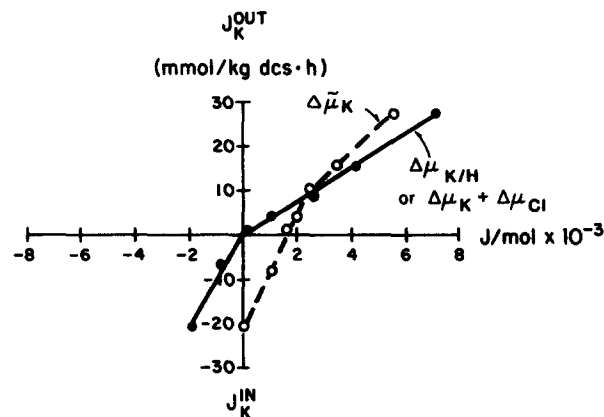


FIGURE 3. NEM-induced net K flux as a function of driving force for conductive  $(\Delta\mu_K + zFE_m)$  or electroneutral  $[(\Delta\mu_K - \Delta\mu_H)$  and  $(\Delta\mu_K + \Delta\mu_{Cl})]$  K loss in isotonic media. The driving force was changed by varying  $[K]_{out}$  ( $\Delta\mu_K$ ) at constant  $[Na + K]_{out}$ . As discussed in the text, valinomycin ( $2 \mu M$ ) was added to all suspensions in order to change  $E_m$ . The membrane potentials in the presence of valinomycin were measured directly with microelectrodes and varied as a function of  $[K]_o$  as previously shown (Cala, 1980, Fig. 4). The NEM-induced net K flux is the difference between the net K flux (measured using cells for the same animal) in NEM plus valinomycin and the K flux measured in valinomycin alone. The valinomycin-induced net K flux obtained its maximal value of 9 mM/kg dcs·h at 3 mM  $K_o$  and was unmeasurable ( $<2$  mM/kg dcs·h at  $>40$  mM  $K_o$ ). The positive values for driving force and flux (upper right-hand quadrant) denote force and flux directed from cell to medium, while the negative values (lower left-hand quadrant) denote flux and force directed from medium to cell. These data are representative of three identical experiments.

function of  $\Delta\mu_K + zFE_m$ , the driving force for conductive K flux, and  $(\Delta\mu_K - \Delta\mu_H)$  or  $(\Delta\mu_K + \Delta\mu_{Cl})$ , the driving forces for electroneutral K/H exchange or KCl cotransport, respectively. The driving forces for conductive and electroneutral K flux were varied by changing the medium K concentration at constant  $[Na + K]$ . The membrane potential was changed by the addition of valinomycin ( $2 \mu M$ ) (see Cala, 1980, Fig. 4) to all media so that  $-\Delta\mu_H$  and  $\Delta\mu_{Cl}$  were no longer equal to  $zFE_m$  (Cala, 1985a). The force-flow plot (Fig. 3) shows that the NEM-induced net K flux ( $J_K^{NEM}$ ) is coupled to  $(\Delta\mu_K - \Delta\mu_H)$  or  $(\Delta\mu_K + \Delta\mu_{Cl})$  and not to  $(\Delta\mu_K +$

$zFE_m$ ). That is,  $J_K^{NEM}$  decreases as a function of the forces driving electroneutral K/H exchange and/or K plus Cl cotransport and is zero when  $(\Delta\mu_K - \Delta\mu_H)$  and  $(\Delta\mu_K + \Delta\mu_{Cl})$  are zero. Most important, the flux reverses direction (changes sign) when  $(\Delta\mu_K - \Delta\mu_H)$  and  $(\Delta\mu_K + \Delta\mu_{Cl})$  change sign. In contrast,  $J_K^{NEM}$  reverses direction (becomes negative), even though the sign of  $(\Delta\mu_K + zFE_m)$  is unchanged (positive). Since the direction (sign) of the flux and the force to which it corresponds must be the same, we conclude that the NEM-induced K flux is electroneutral K/H exchange or K plus Cl cotransport.

*Distinction between K/H Exchange and KCl Cotransport, by Inhibition of Cl/HCO<sub>3</sub> Exchange*

Having established that NEM-induced net K loss is electroneutral K/H exchange or K plus Cl cotransport, we reasoned that it may be possible to distinguish between the two modes of transport on the basis of the K:Cl stoichiometry. That is, if the NEM-activated pathway is K plus Cl cotransport, the "obligatory" stoichiometry between net K and Cl flux should be unity. If net K flux is due to K/H exchange, there should be no obligatory coupling between the net K plus Cl fluxes. The observation that the apparent K:Cl stoichiometry can be  $>1$  when K flux is due to K plus Cl cotransport (Kregenow, 1977) or equal to unity when K flux is mediated by K/H exchange (Cala, 1980, 1985a, b) is a reflection of the parallel operation of Cl/HCO<sub>3</sub> exchange. Briefly, the Cl/HCO<sub>3</sub> exchanger will function to distribute Cl and HCO<sub>3</sub> in accordance with the thermodynamic force that drives the anion exchange ( $\Delta\mu_{Cl} - \Delta\mu_{HCO_3}$ ). As such, any change in  $(\Delta\mu_{Cl} - \Delta\mu_{HCO_3})$  from its steady state value of zero will result in net Cl/HCO<sub>3</sub> exchange flux. Consequently, when K plus Cl cotransport occurs in a membrane with a robust Cl/HCO<sub>3</sub> exchanger, net K loss will exceed that of Cl. This is due to the fact that cell Cl accounts for less than one-half of the intracellular osmolarity; loss of isotonic KCl via K plus Cl cotransport will cause  $[Cl]_i$  to decrease. Therefore,  $\Delta\mu_{Cl}$  will decrease,  $(\Delta\mu_{Cl} - \Delta\mu_{HCO_3})$  will become more negative, and Cl will be driven into the cell through the Cl/HCO<sub>3</sub> exchange. This Cl "backflux" will result in an apparent increase in the K:Cl stoichiometry, above the value of unity expected for K plus Cl cotransport. In analogy with the above example, net H flux via K/H exchange will alter  $[HCO_3]$  and therefore  $\Delta\mu_{HCO_3}$ , which will drive net Cl flux by the Cl/HCO<sub>3</sub> exchanger and give rise to an apparent coupling between K and Cl. In light of the above, we reasoned that it is possible to distinguish parallel K/H and Cl/HCO<sub>3</sub> exchange from parallel K plus Cl cotransport and Cl/HCO<sub>3</sub> exchange by examining the K:Cl stoichiometry in cells where the Cl/HCO<sub>3</sub> exchange is inhibited by DIDS (Cala, 1980, 1985a; Kregenow, 1981). If net K and Cl fluxes are due to the parallel operation of K/H and Cl/HCO<sub>3</sub> exchangers, then inhibition of the Cl/HCO<sub>3</sub> exchange with DIDS should uncouple the net K and Cl fluxes. If, however, K plus Cl cotransport and Cl/HCO<sub>3</sub> exchange pathways are operating in parallel, then inhibition of the anion exchanger should result in a net K:Cl stoichiometry of unity. Fig. 4 depicts net K and Cl loss from NEM-treated cells in the presence and absence of DIDS. While DIDS exerts no significant effect upon the NEM-induced net K flux, there is a 60–70% reduction in the corresponding net Cl flux. This finding

is inconsistent with parallel K plus Cl cotransport and Cl/HCO<sub>3</sub> exchange pathways. Thus, it appears that NEM-induced net K and Cl fluxes are the result of parallel K/H and Cl/HCO<sub>3</sub> exchange.

*Distinction between K/H Exchange and K plus Cl Cotransport*

**Force-flow analysis.** The data in Fig. 3 establish that, like volume-induced K loss, the NEM-induced pathway is electroneutral; however, these data do not permit us to distinguish between K/H exchange and K plus Cl cotransport. While the anion replacement studies would suggest that the NEM- and volume-induced pathways are different, the studies designed to investigate K:Cl stoichiometry (with DIDS) suggest that, like the volume-induced pathway, the NEM-induced K loss is due to K/H exchange. In order to unambiguously determine the nature of the NEM-induced net K flux, studies were designed to distinguish between K/H exchange and K plus Cl cotransport on the basis of thermodynamic criteria.

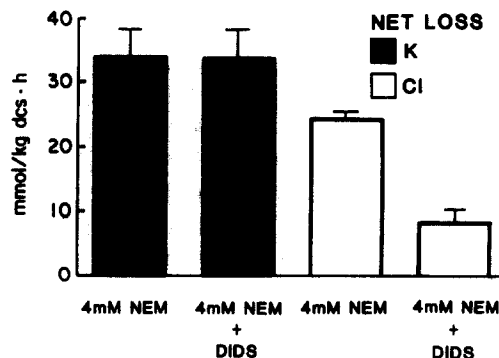


FIGURE 4. Net K and Cl loss from cells in isotonic NEM- (4 mM) containing media in the presence and absence of DIDS (100  $\mu$ M). The data depict net K and Cl loss during 1 h of exposure to NEM with or without DIDS. The data are expressed as the means  $\pm$  SEM of three identical experiments.

Since (as discussed above) H and Cl are distributed at electrochemical equilibrium across the membranes of *Amphiuma* and other red blood cells, it is not possible, under normal circumstances, to distinguish between the forces driving K/H exchange and K plus Cl cotransport. If, however, the Cl/HCO<sub>3</sub> exchanger (which is the primary pathway responsible for establishing equality between  $-\Delta\mu_H$  and  $\Delta\mu_{Cl}$ ) is inhibited, it is possible to independently vary  $\Delta\mu_H$  and  $\Delta\mu_{Cl}$  and therefore  $(\Delta\mu_K - \Delta\mu_H)$  and  $(\Delta\mu_K + \Delta\mu_{Cl})$ . Consistent with this reasoning, the data in Fig. 5 and Table I were obtained from NEM-treated cells where Cl/HCO<sub>3</sub> exchange was inhibited by DIDS and  $\Delta\mu_H$  and  $\Delta\mu_{Cl}$  were varied independently. Fig. 5 presents data obtained from NEM-treated cells where the driving force for K/H exchange was set to zero, while the force driving KCl cotransport was varied from 500 to 6,200 J/mol. Since, regardless of the magnitude of the force driving K plus Cl cotransport, net K flux is zero, as is the force driving K/H exchange, we conclude that NEM-induced K flux is by K/H exchange and not K plus Cl cotransport. The results of similar studies are presented in Table I and show that as  $(\Delta\mu_K - \Delta\mu_H)$  and  $(\Delta\mu_K + \Delta\mu_{Cl})$  are varied (by changing only  $-\Delta\mu_H$

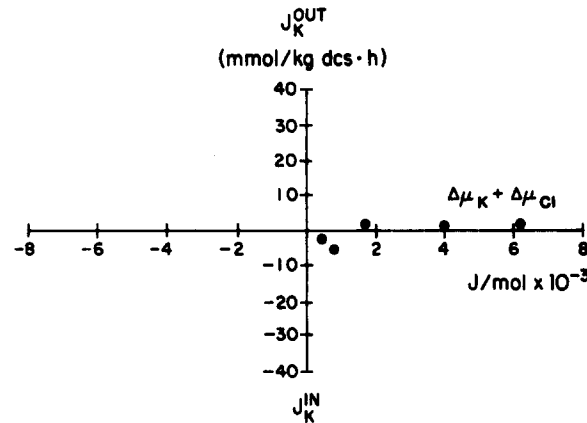


FIGURE 5. Correspondence between the magnitude and direction (sign) of NEM-induced net K flux and the force driving K/H exchange ( $\Delta\mu_K - \Delta\mu_H$ ) or KCl cotransport ( $\Delta\mu_K + \Delta\mu_{Cl}$ ). The cells were treated with 100  $\mu$ M DIDS in order to inhibit the anion exchanger and permit independent variations in  $\Delta\mu_H$  and  $\Delta\mu_{Cl}$ . The force driving KCl cotransport ( $\Delta\mu_K + \Delta\mu_{Cl}$ ) was then varied over a range of 6,000 J/mol while the driving force for K/H exchange was held at zero. These data are representative of three identical experiments. While not shown in the figure, a control that held the force driving KCl cotransport and that driving K/H exchange at  $\sim 7,000$  J/mol resulted in a net K loss of 32 meq in 1 h (see also the legend to Fig. 3).

TABLE I  
Correspondence between the Magnitude and Direction (Sign) of (4 mM)  
NEM-induced Net K Flux and Forces Driving K plus Cl  
Cotransport ( $\Delta\mu_K + \Delta\mu_{Cl}$ ) and K/H Exchange ( $\Delta\mu_K - \Delta\mu_H$ )

pH <sub>out</sub>	[K] <sub>o</sub>	[Cl] <sub>o</sub>	$J_K^{net}$	Net driving force	
				( $\Delta\mu_K + \Delta\mu_{Cl}$ ) J/mol	( $\Delta\mu_K - \Delta\mu_H$ ) J/mol
7.65	113	116	-13.5	-2,026	-2,026
7.65	113	45	-9.5	+393	-2,018
7.10	113	4	+9.0	+6,386	+1,241
7.10	113	116	+6.5	-1,774	+1,189
7.65	113	4	-11.5	+6,407	-2,042

The distinction between K/H exchange and KCl cotransport is based upon the correspondence of flux and driving force. The magnitude and direction (sign) of the driving forces for K/H exchange ( $\Delta\mu_K - \Delta\mu_H$ ) and KCl cotransport ( $\Delta\mu_K + \Delta\mu_{Cl}$ ) were changed independently by varying external Cl ( $\Delta\mu_{Cl}$ ) and/or H ( $\Delta\mu_H$ ) at fixed internal K, Cl, and H. All cells were exposed to 4 mM NEM, 100  $\mu$ M DIDS, and 1 mM ouabain. DIDS was added in order to prevent changes in  $[H]_i$  and  $[Cl]_i$  in response to changes in  $[Cl]_o$  and/or  $[H]_o$  (see text for details).  $Cl_{out}$  was varied by substitution with gluconate. Intracellular pH (7.16) was determined on the lysate of cells that were first frozen and then thawed. Positive values for driving force and/or net K flux correspond to a force or flux directed from cell to media; negative values indicate a flux or force directed from media to cell. The data in the table are representative of two similar experiments. Although the absolute magnitude of the K fluxes varied with different animals, the direction of net K flux always followed the direction of the driving force for K/H exchange ( $\Delta\mu_K - \Delta\mu_H$ ).

and  $\Delta\mu_{\text{Cl}}$ ), the magnitude—but, more important, the direction—of NEM-induced net K flux corresponds with the magnitude and direction of  $(\Delta\mu_{\text{K}} - \Delta\mu_{\text{H}})$  and not  $(\Delta\mu_{\text{K}} + \Delta\mu_{\text{Cl}})$ . Clearly, the NEM-induced K flux by *Amphiuma* red blood cell is by K/H exchange.

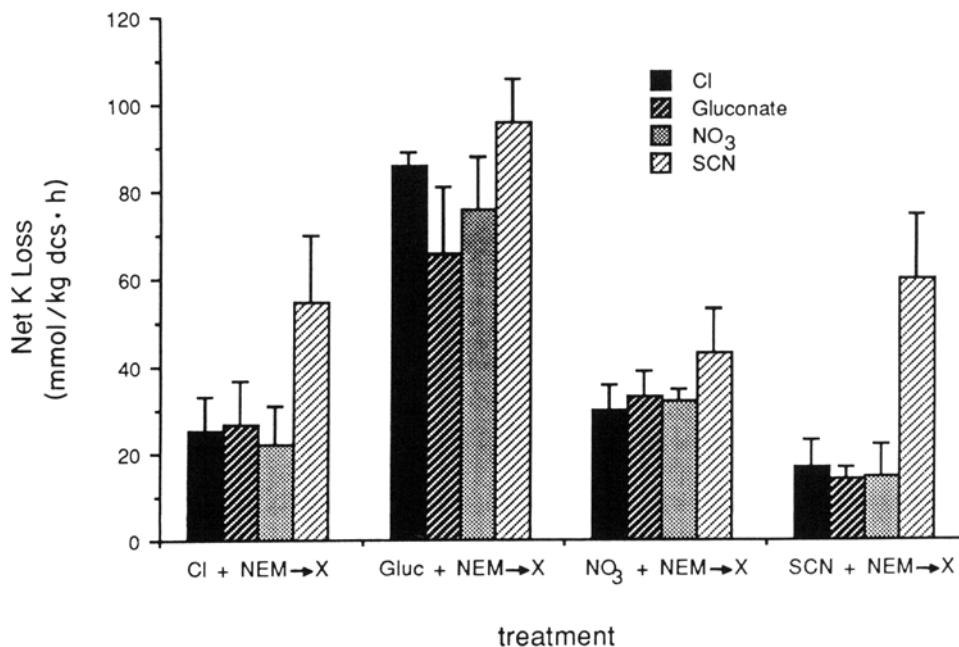
#### *Anion Effects upon NEM-activated K/H Exchange*

**Activation vs. translocation.** Given that the force-flow analyses establish the NEM-induced net K loss as K/H exchange and the fact that previous studies (Cala, 1980, 1983a, b, 1985a) have established volume-induced K loss as K/H exchange, the difference in the response of volume- and NEM-induced K/H exchange to Cl replacement was puzzling. We reasoned that if the K flux pathways are the same, then the differential effects of anions may reflect differences in the means by which K/H exchange is activated (i.e., NEM or volume). That is, the anion effects may reflect differences in anion interactions with the activation step(s) and not ion translocation. In order to test this hypothesis, the studies presented in Fig. 6 were performed. Briefly, cells were suspended in Cl medium or media in which Cl was replaced with a test anion (Cl, NO<sub>3</sub>, gluconate, or SCN). Subsequently, the cells were exposed to NEM (4 mM) for 45 min and then transferred to NEM-free media containing either the anion present during NEM activation or one of the test anions, and net K/H exchange flux was measured. The results show that if cells are exposed to NEM in Cl medium and then transferred to Cl, NO<sub>3</sub>, or gluconate medium, the flux (NEM-induced) is what would be seen if Cl were always present, and yet K flux is stimulated in SCN. Similarly, when cells are treated with NEM in gluconate (an anion that is stimulatory relative to Cl and NO<sub>3</sub>), the subsequent suspension of cells in NEM-free gluconate, Cl, NO<sub>3</sub>, or SCN media results in K/H exchange fluxes that are typical of cells activated by NEM in gluconate. The K/H exchange fluxes by cells activated by NEM in NO<sub>3</sub> and subsequently transferred to NO<sub>3</sub>, Cl, or gluconate media are essentially the same and resemble the K fluxes that would be observed if NO<sub>3</sub> were always present. K loss from cells activated by NEM in NO<sub>3</sub> medium is stimulated upon transfer to NEM-free SCN medium. When cells are activated by NEM in SCN medium and then transferred to Cl, NO<sub>3</sub>, or gluconate, the K fluxes measured in the presence of all three of these anions are equal but smaller than that in the presence of SCN.

(Note: NEM does not inhibit net anion exchange. Thus, with the exception of gluconate, the anion present during the flux period [after NEM pretreatment] is present both inside and outside the cell. Since gluconate is not transported on the anion exchanger, cells transferred to gluconate medium gain HCO<sub>3</sub> and lose Cl, NO<sub>3</sub>, or SCN, while transfer from gluconate to Cl, NO<sub>3</sub>, or SCN results in a loss of HCO<sub>3</sub> in exchange for these anions [see legend to Fig. 2]. These studies differ from those described previously [see Figs. 1–5] in that the flux media used in the present studies contained no NEM.)

In summary, when exposed to NEM in NO<sub>3</sub>, Cl, or gluconate media and then transferred to NEM-free NO<sub>3</sub>, Cl, or gluconate, cell K loss resembles that which would be seen if the anion present during NEM exposure were also present during the flux measurement period. As such, it appears that the effects of Cl,

$\text{NO}_3$ , and gluconate on NEM-induced net K loss (Figs. 2A and 6) reflect effects upon activation and not translocation. In contrast, SCN stimulates K loss regardless of the anion present during NEM exposure. Thus, the effects of SCN are complex and different from those of the other anions studied. Taken together, the effects of Cl,  $\text{NO}_3$ , and gluconate suggest interactions of these anions with K flux activation. When viewed together with the SCN data, it is clear that anion



**FIGURE 6.** Net K loss from cells activated by NEM in isotonic Cl, gluconate,  $\text{NO}_3$ , or SCN media before experimentation (flux measurement) in isotonic NEM-free Cl, gluconate,  $\text{NO}_3$ , or SCN media. The protocol was to suspend cells in isotonic media containing one of the above anions for 15 min in order to deplete cell Cl and load cells with the test anion before exposure to NEM. Since gluconate is not transported on the  $\text{Cl}/\text{HCO}_3$  exchanger, the cells in gluconate gained  $\text{HCO}_3$  and became alkaline, with the internal pH increasing from a normal value of 7.00 to a final value of 7.35. After exposure to the test anion (Cl, gluconate,  $\text{NO}_3$ , or SCN) for 15 min, the cells were transferred to medium containing the same anion plus 4 mM NEM for 45 min. The K concentration of the NEM-containing medium was increased so that the cell K content and concentration were unchanged despite the presence of NEM. To begin the experiment, the cells were divided into equal aliquots and transferred to NEM-free isotonic Cl, gluconate,  $\text{NO}_3$ , and SCN media. While the data in Figs. 1–5 were obtained from cells in NEM-containing media, the ion flux data in the present figure were obtained from cells preincubated (activated) in NEM but studied in NEM-free media. These experiments differ from those previously presented in that NEM was present during the flux measurements described in Figs. 1–5, but only during the preincubation or activation period of cells from which data were obtained for this figure.

effects upon inducible transport pathways cannot be assumed simply to reflect substrate or co-factor effects on the translocation step.

#### DISCUSSION

The present studies show that, as has been reported for low-K sheep erythrocytes, millimolar NEM stimulates net K loss by *Amphiuma* red blood cells. Further, these studies show that the magnitude of net K flux is dependent upon the NEM concentration. Studies evaluating the K:Cl stoichiometry suggested the NEM-induced K loss is via K/H exchange. Direct proof that NEM induces K/H exchange in *Amphiuma* red blood cells under isosmotic conditions was obtained using a force-flow analysis. In contrast, the more model-dependent anion replacement studies failed to identify the mode of the NEM-induced net K flux.

The results of kinetic and pharmacological studies can be consistent with but not necessarily unique to a particular mode of transport (see Cala, 1983a, b, 1985a, b). In the present study, we designed experiments to distinguish between K conductance, K/H exchange, and K plus Cl cotransport based upon the forces to which each type of transport must couple. Since K flux by all pathways is dependent upon the potential energy stored in the K gradient (diffusional drift),  $\Delta\mu_K$  is a term common to all expressions for the force driving K. As such, our analysis focused upon the correspondence between net K flux and the potential energy term responsible for the imposed drift of K. The terms that describe the energy contributing to the imposed drift of K owing to conductive flux, electroneutral K/H exchange, or K plus Cl cotransport are  $zFE_m$ ,  $-\Delta\mu_H$ , and  $\Delta\mu_{Cl}$ , respectively. In Fig. 3 (negative quadrants), net K flux is into the cell, while  $\Delta\mu_K + zFE_m$  favors net K loss by conductive routes. In contrast,  $-\Delta\mu_H$  and  $\Delta\mu_{Cl}$  are of sufficient magnitude and in such a direction as to favor electroneutral K uptake. Consequently, we conclude that net cellular K uptake by NEM-treated cells must be due to either K/H exchange or K plus Cl cotransport. Having determined that the NEM-induced K flux is either by K/H exchange or K plus Cl cotransport, we needed to determine whether  $\Delta\mu_H$  or  $\Delta\mu_{Cl}$  is a contributor to the force driving net K transport. In order to evaluate the contribution of  $\Delta\mu_H$  or  $\Delta\mu_{Cl}$  to net driving force, experiments were performed upon cells in which the Cl/HCO<sub>3</sub> exchange was inhibited by DIDS so that  $\Delta\mu_H$  and  $\Delta\mu_{Cl}$  could be varied independently. The data in Fig. 5 and Table I show that net K flux is independent of  $(\Delta\mu_K + \Delta\mu_{Cl})$  and therefore of  $\Delta\mu_{Cl}$ . Thus, we concluded that NEM-induced K flux is the result of K/H exchange and not K plus Cl cotransport.

#### *Effects of Anions*

Relative to Cl, NO<sub>3</sub> and SCN were found to inhibit K loss from swollen cells but stimulate K loss from cells in the presence of NEM (see Fig. 2). Because both osmotic swelling and NEM treatment activate K/H exchange in *Amphiuma* red blood cells, the data of Fig. 2 deserve further discussion. Implicit in the interpretation of the anion replacement studies is the assumption that anion effects are referable to the role of anions as (a) electrical counterions, and/or (b) substrates. Since both volume- and NEM-activated K fluxes are due to electroneutral K/H exchange, this assumption does not pertain. That is, since fluxes are electroneu-

tral, the presence of an electrical counterion is irrelevant. Further, because Cl is not a species transported by the K/H exchanger, Cl is, by definition, not a substrate. Consequently, the differences in anion sensitivities of volume- and NEM-induced K/H exchange (Fig. 2, A and B) raised questions regarding the use of anions as probes for the volume- and NEM-sensitive alkali metal ion flux pathways in *Amphiuma* red blood cells.

*The Stimulatory Effects of Anions on K/H Exchange: Considerations of Cell Volume and pH*

Alkali metal/H exchange flux by *Amphiuma* red blood cells is both pH and volume sensitive (Cala, 1980, 1983a, 1985b). Therefore, replacing medium Cl with other anions can cause changes in cell volume and pH, thereby altering the rate and magnitude of alkali metal/H exchange flux in ways that are only indirectly related to anion composition. For example, the *Amphiuma* red blood cell has a robust Cl/HCO<sub>3</sub> exchanger that will mediate net Cl/HCO<sub>3</sub> exchange until these ions have the same transmembrane distribution. Consequently, replacing external Cl with an anion that is not transported or is transported slowly by the anion exchanger will cause net Cl efflux and HCO<sub>3</sub> influx, altering both cell volume and pH. That is, the cell interior is alkalinized owing to HCO<sub>3</sub> uptake in exchange for cell Cl. Because  $P_{\text{CO}_2}$  is constant and the cell and medium buffer capacities are high, the increase in cell [HCO<sub>3</sub>] is smaller than the decrease in cell [Cl] and the cells shrink. In the present studies, cell volume is not a variable. Since shrinkage of *Amphiuma* red blood cells activates an Na/H exchanger that restores the cells to control volume, cell shrinkage resulting from replacing Cl<sub>o</sub> with a nonexchangeable anion is compensated for by the above volume-regulatory mechanism. However, because volume regulation by shrunken cells is achieved as a result of Na uptake in exchange for cell H, the volume-regulatory Na/H exchanger further alkalinizes the cell interior. Thus, in the present studies, while volume is not a variable, it is necessary to distinguish between effects caused by anions per se and those that are secondary to changes in pH.<sup>1</sup> We have previously reported (Cala, 1980, 1983b) that the *Amphiuma* red blood cell K/H exchanger is stimulated by alkalinizing the cell interior. Further, gluconate, which cannot move on the anion exchanger, causes the cell interior to alkalinize by ~0.3–0.4 pH units as the cells lose Cl in exchange for HCO<sub>3</sub>. Consequently, the increased K loss by osmotically swollen and NEM-treated cells in gluconate media is most probably a result of intracellular alkalinization. This interpretation was corroborated by studies (data not presented) that showed that suspension of cells in isosmotic sucrose media (where there is no external anion

<sup>1</sup> Replacing medium Cl with gluconate results in a pH<sub>i</sub> increase of 0.3–0.4 pH units (normal pH<sub>i</sub> = 7.0) and causes the cells to shrink below control volume. Subsequent volume recovery (Na/H exchange) after the gluconate-induced cell shrinkage results in a further increase in pH<sub>i</sub> of 0.15–0.2 units. In control experiments performed upon cells in isotonic gluconate (data not shown), the alkalinization due to Na/H exchange after shrinkage was avoided. The NEM-induced K losses from cells in isosmotic (pH<sub>i</sub> = 7.55) or isotonic (pH<sub>i</sub> = 7.35) gluconate was not different. Thus, it appears that above pH 7.35, further alkalinization is without effect upon NEM-induced K loss.



other than  $\text{HCO}_3^-$ , which can exchange for cell Cl) caused a change in intracellular pH similar to that in gluconate and resulted in an equivalent stimulation of K/H exchange. More modest changes in cell pH resulted when cells were suspended in media containing PAH. Cells suspended in PAH-containing media initially alkalize (to the same extent as cells in gluconate media) as cell Cl is exchanged for external  $\text{HCO}_3^-$ . The rapid initial alkalization is followed by a slow return (90–120 min) of the cell pH to near control levels as the previously gained  $\text{HCO}_3^-$  leaves the cell in exchange for external PAH (Motaïs, 1977). The pH of the PAH-treated cells was  $\sim 0.1$  pH unit greater than controls at the time of the flux experiments (Fig. 2). Thus, the increased K loss by NEM-treated cells in media containing PAH is probably due to cell alkalization. At present, we cannot explain the lack of effect of PAH on the K flux from osmotically swollen cells. Because neither cell volume nor pH was a variable for cells in media containing SCN or  $\text{NO}_3^-$ , the stimulatory effects of these anions on the K flux of NEM-treated cells (Fig. 2A) cannot be attributed to changes in cell pH or volume.

#### *Volume- and NEM-activated K/H Exchange*

As shown above, both volume- and NEM-activated K loss by *Amphiuma* red blood cells are by K/H exchange. Further, the NEM- and volume-induced K/H exchange fluxes are nonadditive and have similar H sensitivities. With regard to the latter point, previous studies have shown that the inward-directed rectification (negative quadrant of force-flow plots; Figs. 3 and 5) of volume-activated K/H exchange is a substrate effect of H, reflecting the asymmetry in the transmembrane  $[\text{H}^+]$  distribution  $[\text{H}]_i/[\text{H}]_o = \sim 3\text{--}4$  (see Cala, 1985b). While not dealt with explicitly, inward-directed rectification of K/H exchange flux can be seen in Fig. 3. Taken together, the above observations are consistent with the interpretation that both volume- and NEM-activated K/H exchanges occur by the same translocation mechanism.

#### *Effects of Anions*

If we assume that the translocation is the same regardless of its means of activation (volume or NEM exposure), it seems reasonable to conclude that  $\text{NO}_3^-$  and SCN inhibition of K flux by swollen cells and stimulation of K flux by NEM-treated cells do not reflect interactions with the translocation step. That is, the difference in the  $\text{NO}_3^-$  and SCN sensitivity of K/H exchange by swollen and NEM-treated cells is not due to differences in the K flux pathway. Since one obvious difference between volume- and NEM-induced K/H exchange flux is the means of activation, it seems reasonable that differences in anion sensitivity reflect differences in the anion sensitivity of the activation and not the translocation process. In support of this hypothesis are the data presented in Figs. 2A and 6. When cells are transferred from isotonic Cl medium to NEM-containing media in which Cl,  $\text{NO}_3^-$ , SCN, gluconate, or PAH is the sole anion, K/H exchange flux is stimulated relative to Cl (Fig. 2A). If, as shown in Fig. 6, cells are exposed to NEM in Cl medium and then transferred to isotonic NEM-free Cl, gluconate, or  $\text{NO}_3^-$ , K/H exchange fluxes are equal to those in Cl medium. The same is true for cells exposed to NEM in gluconate or  $\text{NO}_3^-$  media. These data, which show that the

NEM-induced net K/H exchange flux is dependent upon the anion present during NEM treatment (activation), but independent of media composition after NEM exposure, suggest that the effects of anions are on the activation and not the ion translocation steps. The data obtained from cells exposed to SCN during or after NEM activation of K/H exchange do not fit this pattern, which suggests that SCN exerts multiple effects.

The conclusion that anion effects are upon activation and not translocation gains support from previous studies of a similar nature performed upon dog red blood cells (Parker, 1984). Parker was able to activate Na/H exchange and prevent volume-sensitive inactivation (sustain activation) by "fixing" the activated cells with glutaraldehyde. Subsequently, he was able to show that SCN, which inhibits Na/H exchange by cells shrunken in the presence of SCN, is not inhibitory to Na/H exchange in glutaraldehyde-fixed cells. Thus, he concluded that SCN does not exert its inhibitory effects directly upon the Na/H exchanger but on some steps responsible for activation.

In summary, we have shown that millimolar NEM activates net K loss by *Amphiuma* red blood cells in isotonic medium. Using force-flow analysis, we have designed experiments that permit us to isolate the terms responsible for imposed drift if K flux is by K/H exchange ( $\Delta\mu_H$ ), K plus Cl cotransport ( $\Delta\mu_{Cl}$ ), or conductive transport ( $zFE_m$ ). Analysis of such studies shows that the NEM-induced net K loss is due to electroneutral K/H exchange. Studies employing anion replacement as a means of identifying the K flux pathway were ambiguous since the effects of anion replacement were related to the activation and not translocation. The above ambiguities point out the difficulties involved in interpreting the effects of manipulations performed upon inducible transport pathways. That is, when dealing with non-inducible systems (i.e., the human red blood cell anion exchange), manipulations that alter transport rate and magnitude can, in the absence of membrane disruption, be interpreted in terms of effects directly upon the ion translocation processes (carrier). In contrast, when manipulations are performed that alter transport by inducible transport pathways (i.e., the K/H exchange in the present study), care must be taken to distinguish effects upon the induction mechanism from those that are directly upon translocation.

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